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Research Article

We have recently demonstrated that all-trans retinoic acid (RA), the active metabolite of vitamin A, is an efficient alternative to chemotherapy in the treatment of acute promyelocytic leukemia (AML3). We have further shown that, in these AML3 cells, the gene of the retinoic acid receptor-alpha (RAR alpha) is translocated from chromosome 17 to chromosome 15, and fused to a new gene, PLM. This results in the expression of both normal and chimeric RAR alpha transcripts in AML3 cells. The PLM-RAR alpha protein may account for the impairment of differentiation and thus leukemogenesis, but not for the paradoxical efficacy of RA in these cells. In an attempt to elucidate RA's differentiative effect in AML3 patients, the present work examined the in vitro and in vivo modulation of the normal RAR alpha transcripts by all-trans RA in seven cases of AML3. In all samples, Northern blot analysis revealed a low expression of the two normal RAR alpha transcripts compared with other human myeloid leukemic cells. No modulation was observed after 4-8 d of in vivo therapy with all-trans RA 45 mg/m2 per d. In vitro incubation with all-trans RA, however, increased the level of expression of the normal RAR alpha transcripts in AML3 cells but not in other AML leukemic subtypes. This modulation of the two normal RAR alpha transcripts appeared to […]

All-*trans* Retinoic Acid Modulates the Retinoic Acid Receptor-α in Promyelocytic Cells

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Abstract

We have recently demonstrated that all-*trans* retinoic acid (RA), the active metabolite of vitamin A, is an efficient alternative to chemotherapy in the treatment of acute promyelocytic leukemia (AML3). We have further shown that, in these AML3 cells, the gene of the retinoic acid receptor-α (RARα) is translocated from chromosome 17 to chromosome 15, and fused to a new gene, PLM. This results in the expression of both normal and chimeric RARα transcripts in AML3 cells. The PLM-RARα protein may account for the impairment of differentiation and thus leukemogenesis, but not for the paradoxical efficacy of RA in these cells. In an attempt to elucidate RA's differentiative effect in AML3 patients, the present work examined the in vitro and in vivo modulation of the normal RARα transcripts by all-*trans* RA in seven cases of AML3. In all samples, Northern blot analysis revealed a low expression of the two normal RARα transcripts compared with other human myeloid leukemic cells. No modulation was observed after 4–8 d of in vivo therapy with all-*trans* RA 45 mg/m^2 per d. In vitro incubation with all-*trans* RA, however, increased the level of expression of the normal RARα transcripts in AML3 cells but not in other AML leukemic subtypes. This modulation of the two normal RARα transcripts appeared to be an early and primary event of RA's differentiating effect. We therefore suggest that up-regulation of the normal RARα gene expression by pharmacological concentrations of all-*trans* RA may restore the normal differentiation pathway in these cells. (*J. Clin. Invest.* 1991. 88:2150–2154.) Key words: acute promyelocytic leukemia • differentiation • receptor regulation • retinoids

Introduction

Acute promyelocytic leukemia (AML3),[1] the M3 subtype of the FAB cytological classification (1), is characterized by the accumulation and/or proliferation of cells blocked in their myeloid differentiation. Almost all the cases have a specific chromosomal translocation in the leukemic clone, identified as a reciprocal translocation between chromosomes 15 and 17, referred to as t(15;17) (2). We and others (3–6) have recently reported that, through this chromosome exchange, the retinoic acid receptor-α (RARα)[1] gene of chromosome 17 is brought under the control of a newly defined gene *myl*, renamed PLM (3), on chromosome 15. This new genomic configuration results in one allele of the RARα gene being rearranged, generating abnormal RARα transcripts, with the two normal RARα transcripts still being expressed (5, 6). The PLM-RARα fusion product appears functionally altered, suggesting that impairment of differentiation may lead to leukemogenesis. Paradoxically, we have previously reported that these AML3 leukemic cells are specifically responsive to all-*trans* retinoic acid (RA). In vitro, retinoids induce specific terminal differentiation to mature granulocytes (7), and in vivo, oral all-*trans* RA therapy allows to obtain complete remission in AML3 patients (8–11). No study has so far approached the molecular basis of all-*trans* RA efficacy in these patients. The high expression of RARα in normal differentiated granulocytes (6) and the effect of RA on normal myeloid progenitor cells (12) has led us to postulate that RA and its receptor α may play a role in granulocytic differentiation. We therefore initiated a study of the in vitro and in vivo modulation of the normal RARα gene expression in RA-treated AML3 cells. We report an increase of the normal RARα gene expression in all the RA-treated AML3 cells tested. This appears as an early event of RA and forwards an explanation for the paradoxical effect of all-*trans* RA in this disease.

Methods

Cell samples, cultures, and differentiation assays. Bone marrow or blood samples from seven patients with AML3 were collected in heparinized tubes. Diagnoses were performed according to the FAB cytological classification criteria (1) (Table I). Mononuclear cells were removed from the interface after Ficoll-Hypaque (Eurobio, Paris, France) density gradient. The leukemic cell fraction was enriched after monocyte adherence to plastic surfaces. The leukemic cells of five patients were then immediately prepared for differentiation assay in the presence or absence of all-*trans* RA 10^{-6} M (kindly provided by Dr. W. Bollag, Hoffman-Laroche Laboratories, Basel, Switzerland) as already described (7). Cell viability was assessed by the trypan blue exclusion dye test. The extent of induced differentiation was determined by morphology, and functional criteria as described (7). Cells from myeloid human leukemic cell lines (HL-60, NB4) (gift of T. R. Breitman, National Institutes of Health, Bethesda, MD, and M. Lanotte, Unitè INSERM 301, Paris, France), were maintained in RPMI 1640 supplemented with 15% fetal calf serum, antibiotics, and glutamine. Actinomycin D and cycloheximide were obtained from Sigma Chemical Co., St. Louis, MO.

Nucleic acid extraction and electrophoresis. Total RNA (15 μg per lane) was electrophoresed on a 1.1% agarose-formaldehyde gel after

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1. *Abbreviations used in this paper:* AML3, acute promyelocytic leukemia; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RA, retinoic acid; RARα, retinoic acid receptor-α.

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Table I. Hematological and RA In Vitro Differentiating Efficacy Data of the AML Patients and Cell Line Studied for RARα Gene expression

		Incubation						
			24 h		72 h		5 d	
Patient	FAB*	0 h	C	RA	C	RA	C	RA
			%					
1	AML3	0‡	1	24	3	61	3	85
2	AML3	0	ND§	ND	1	97	13	100
3	AML3	0	ND	ND	7	57	3	98
4	AML3	0	ND	ND	0	95	1	100
5	AML3	0	ND	ND	2	68	4	98
6	AML3	0	ND	ND	1	87	0	95
7	AML3	0	ND	ND	0	90	1	100
8	AML1	0	ND	ND	9	5	10	9
NB4	AML3	0	1	15	0	85	1	100

* FAB morphological classification (1). ‡ Percentage of differentiated cells estimated according to de Thé et al. (3), in the absence (C) or presence (RA) or all-*trans* RA 10^{-6} M. § ND, not done.

denaturation in formamide loading buffer. After electrophoresis, RNA fragments were transferred onto a nitrocellulose filter (Hybond C, Amersham, Bucks., UK) and hybridized to RAR-specific probes as previously described (6). A rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to rehybridize the filters and normalize RNA expression.

Results

RARα gene transcripts are induced during granulocytic differentiation in M3 patients. We first determined the effects of all-*trans* RA on the expression of the normal RARα transcripts during in vitro granulocytic differentiation of fresh AML3 cells. Fresh hematopoietic samples from five patients with AML3 were cultured in the presence of all-*trans* RA 10^{-6} M for 5 d. Cell aliquots were taken at different times of incubation. Cell viability, morphology, degree of functional differentiation, and RARα expression were analyzed and compared to the control samples. Northern blot analysis of these AML3 cell cultures using a RARα-specific cDNA probe is shown in Fig. 1. The presence of abnormal transcripts has already been described in M3 patients (3, 5, 6). The number of abnormal transcripts per sample varied from 1 to 4 and their intensity, relative to their normal counterparts, was variable. In control-untreated samples, the two normal RARα transcripts of 3.2 and 2.3 kb were detected. After normalization for GAPDH mRNA, the levels of expression of these transcripts were found significantly lower in these cells when compared with other leukemic cells (Fig. 1, *AML1* and data not shown). The level of expression of the smaller 2.3-kb transcript was particularly low in patients 2 and 3 (Fig. 1, lanes marked *1*). Incubation with all-*trans* RA 10^{-6} M strikingly increased the level of expression of both transcripts in all the AML3 samples tested as early as after 24 h of incubation (5-fold for the 3.2-kb transcript and 10-fold for the 2.3-kb transcript). This increase in the level of expression persisted after 3 d of incubation (Fig. 1, *right*). All the AML3 samples tested showed significant differentiation criteria in the presence of all-*trans* RA 10^{-6} M (Table I). Leukemic cells from other AML subtypes showed no modification of the RARα transcripts and no functional differentiation criteria after incubation with RA (Fig. 1 and Table I and data not shown).

Study of the RARα expression was also determined in M3 patients under all-*trans* RA therapy. RNA was extracted from

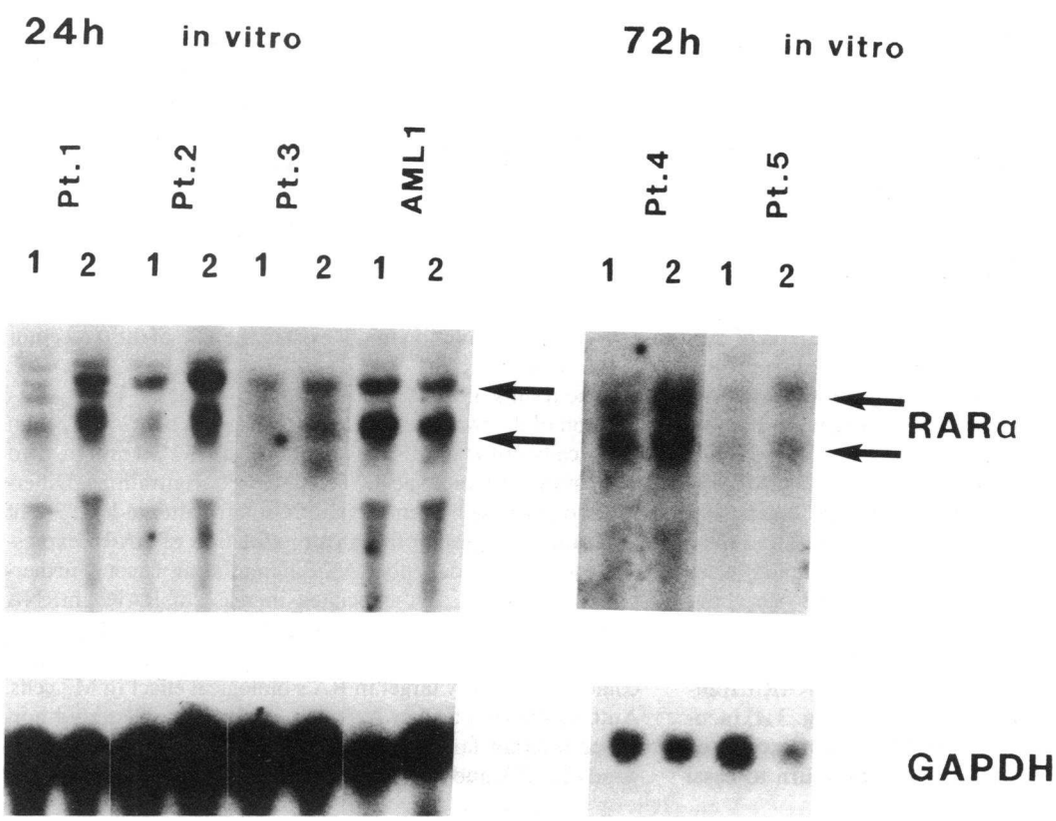

Figure 1. Analysis of RARα gene expression in RNA from leukemic hematopoietic cells treated in vitro with all-*trans* RA. Total RNA (15 μg) from fresh human leukemic cells treated in vitro with all-*trans* RA 10^{-6} M for 24 h, patients (*Pt.*) *1–3*—AML3 patients and one patient with an AML1—and for 72 h, patients *4* and *5*. Short arrows point to the normal transcripts. Lane *1*, control samples; Lane *2*, RA-treated samples.

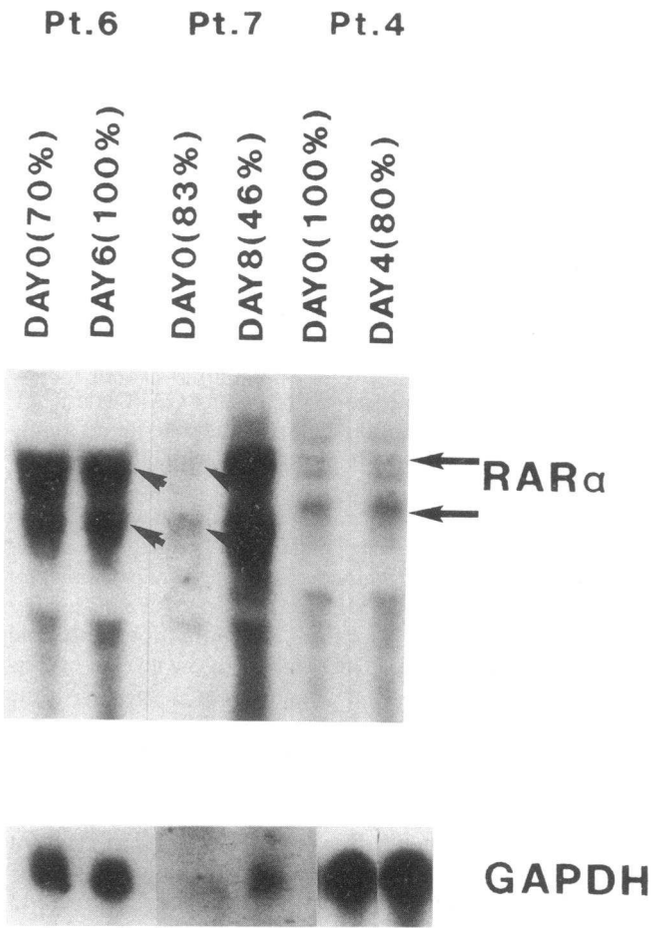

Figure 2. Analysis of RARα gene expression in RNA from mononuclear cells of M3 patients treated with all-*trans* RA 45 mg/m^2 per d. Days of in vivo treatment with all-*trans* RA are indicated. Percentage of leukemic cells in the samples is shown in parentheses. Short arrows point to the normal transcripts.

leukemic cells taken after 4 or 8 d of therapy with all-*trans* RA and was electrophoresed together with RNA extracted from the cells obtained from the same patient before therapy. During 4–8 d of in vivo treatment with all-*trans* RA 45 mg/m^2 per d, no significant modification of the RARα expression was noted (Fig. 2). At this time the bone marrow blast infiltration is still homogeneous, and contrary to in vitro data, no criteria of differentiation are apparent (5). In agreement with our previous studies (6, 13) no expression of the RARβ gene was found in any of these cell samples (data not shown), and no induction was observed after RA incubation.

Modulation of the RARα expression in RA-sensitive promyelocytic leukemic cells. To further study the kinetics of RARα by RA in AML3 cells, cells of the NB4 cell line were incubated with all-*trans* RA 10^{-6} M and cell aliquots were taken at different times. The leukemic cell line NB4 bears the abnormal RARα transcript (3) and differentiates in the presence of all-*trans* RA 10^{-6} M (14) (Table I). A progressive increase of the level of expression of the normal RARα transcripts was significantly observed after 30 min (Fig. 3 *A*) incubation, stable at 24 h (Fig. 3 *B*) and after correction for GAPDH expression, there was a tendency to return to basal levels after 3 and 6 d in culture. The abnormal 4-kb transcript of NB4 cells does not appear modulated in the presence of RA. When the NB4 cells were treated with different concentrations of all-*trans* RA for 5 h, the stimulatory effect was maximal between 10^{-5} and 10^{-7} M, which correspond to in vitro differentiating concentrations (Fig. 3 *B*).

In order to determine whether this RA-dependent induction of RARα gene expression is associated with transcriptional activation and/or an enhanced stabilization of the RARα transcripts, NB4 cells were first treated with RA for 5 h in the absence or presence of cycloheximide. The inhibition of protein synthesis had no detectable effect on RARα expression; in particular, it did not affect the increase of RARα expression induced by all-*trans* RA (compare lane *CH* with *C* and lane *5h* with *RA* + *CH* in Fig. 3 *B*). On the other hand, treatment of the cells with actinomycin D completely inhibited the expression of RARα in control and RA-treated NB4 cells (Fig. 3*B*).

Discussion

Terminal differentiation of acute myeloid leukemic cells has opened both new perspectives on the understanding of leukemogenesis, and new possibilities of therapies in malignancy. To date, in vitro and in vivo differentiation of ALM3 cells with all-*trans* RA is the first model of differentiation therapy (8–11). Distinct RARs have been molecularly cloned (15–20). We and others have identified the specific expression of normal and abnormal RARα transcripts in ALM3 and abnormal RARα's have been identified (3–6). The abnormal transcripts are under the regulation of a newly identified gene PLM from chromosome 15. Keeping in mind the strong implication of RA and RARα in normal (6, 7, 12) and leukemic (21) granulocytic differentiation, an altered RARα may account for the differentiation arrest observed in these cells and be one of the steps leading to their leukemogenesis. The presence of abnormal transcripts has already been described in M3 patients (6, 9). The number of abnormal transcripts per sample varied from 1 to 4 and their intensity, relative to their normal counterparts, was variable. However, the paradoxical efficacy of all-*trans* RA in these cells remains unexplained. The presence of normal RARα transcripts in M3 cells proposes the normal RARα as a possible candidate receptor for mediating RA-induced granulocytic differentiation. Because autoregulation of RAR by its ligand was noted for the β receptor gene (13), we postulated that a similar autoregulation might exist in the case of the α receptor of RA in AML3 cells.

The results of this study favor this hypothesis. The low expression of the two normal RARα transcripts of 3.2 and 2.3 kb specifically noted in M3 cells is increased after 30 min in vitro incubation with biologically active RA concentrations. Differentiation criteria become apparent in vitro after at least 3 d of incubation, suggesting that this modulation of RARα expression is not secondary to RA's differentiating effect. Furthermore, in that the RA-associated increase of RARα mRNA level is independent of protein synthesis and inhibited by inhibitors of RNA synthesis in the AML3 cell line, the RARα gene could be a primary target in RA's biological effect in M3 cells. Autoregulation of other members of the thyroid/steroid hormone receptor family has been shown in response to their ligand (23, 25), and evidence has been brought forward to show

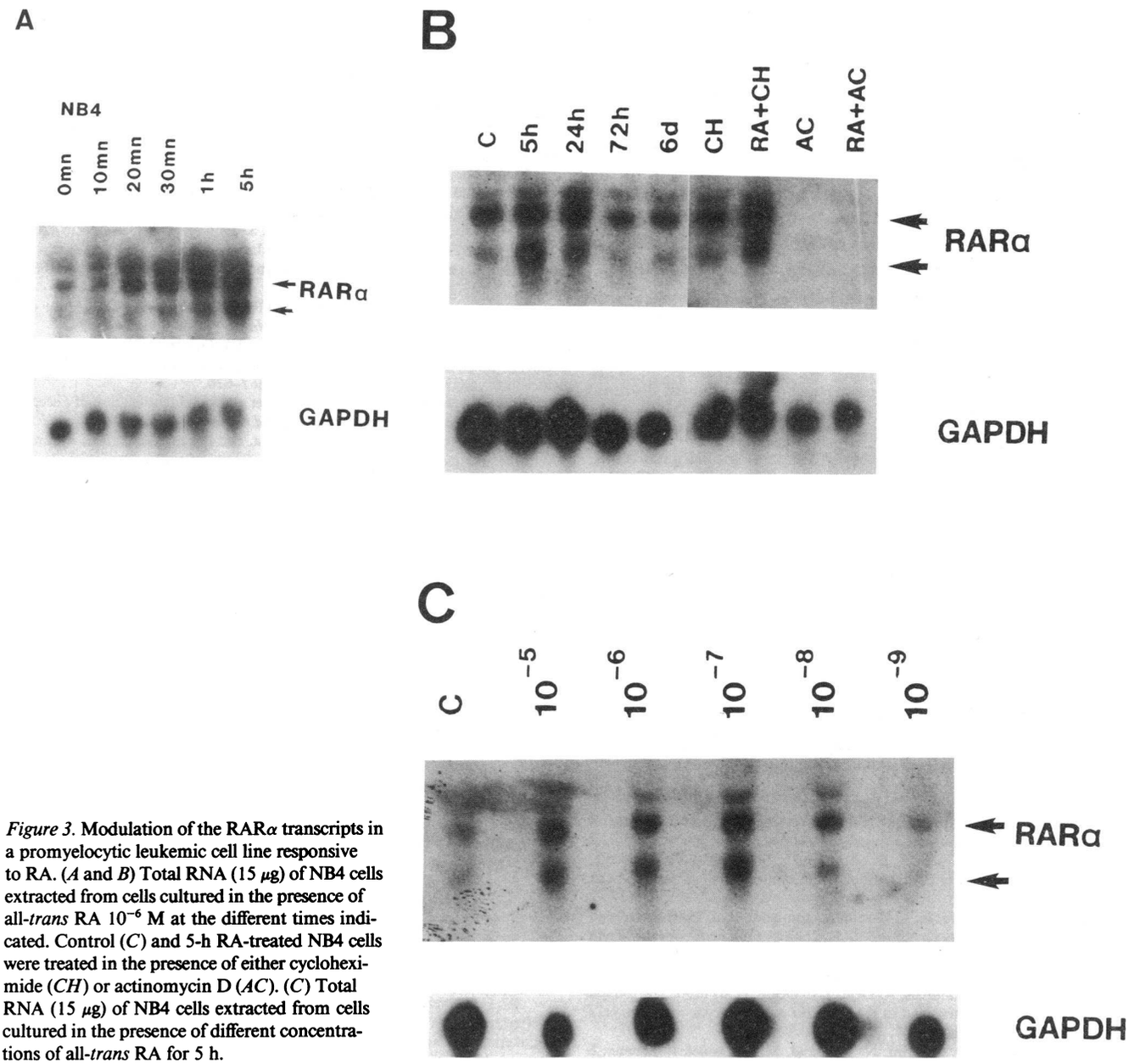

Figure 3. Modulation of the RARα transcripts in a promyelocytic leukemic cell line responsive to RA. (*A* and *B*) Total RNA (15 μg) of NB4 cells extracted from cells cultured in the presence of all-*trans* RA 10^{-6} M at the different times indicated. Control (*C*) and 5-h RA-treated NB4 cells were treated in the presence of either cycloheximide (*CH*) or actinomycin D (*AC*). (*C*) Total RNA (15 μg) of NB4 cells extracted from cells cultured in the presence of different concentrations of all-*trans* RA for 5 h.

that, for some of these ligands, this appeared at a transcriptional level (23, 25). We have described an RA-inducible promoter in the RARβ gene (22), but to date no RA-inducible promoter has been found in the RARα gene and other regulation pathways of the RARα messenger RNA may therefore be involved.

After 4–8 d of in vivo treatment with all-*trans* RA, no such modification is observed. This might be explained by a shut-off of the modulation after 3 d of treatment, as observed in vitro. The kinetics of differentiation are much slower in vivo (median of 30 vs. 6 d), and thus it is probable that at the tested times, biological parameters, such as intranuclear concentration of all-*trans* RA or another active metabolite, for example, are not met in all the M3 cell population. However, we have previously reported that the observed in vitro differentiating effect of all-*trans* RA in short-term cultures was correlated to the therapeutic effect (5), suggesting that the up-regulation of RARα transcripts might also be observed in vivo, but with a different timing.

Our results suggest that in vitro the RARα expressed in AML3 cells may be induced by RA. This is the first report of RA-induced modulation in human myeloid leukemic cells sensitive to RA. In HL-60 or U-937 myelomonocytic leukemic cells, which have no rearranged RARα gene, the steady-state levels of the two RARα transcripts are not affected by induction of terminal differentiation either to granulocytic or monocytic cells (26–28). Regulation of receptors of the thyroid/steroid hormone superfamily has been shown to be cell specific (29, 30) and it is important to note, that, compared to the HL-60 and U-937 myeloid RA-sensitive leukemic cells, the M3 cells represent a specific leukemic cell model. AML3 cells bear one or several abnormal transcripts resulting from the fusion of

the RARα and PLM genes through the t(15;17) translocation. The presence of the chimeric RARα-PLM gene product in these cells may repress (directly or indirectly) the RA target genes. This repression might be overcome by pharmacological concentrations of RA that would restore functional levels of the RARα transcripts, target gene activation, and granulocytic differentiation.

Our results on the RARα transcript modulation in M3 patients who have responded to RA therapy suggests that the induction of a normal expression of the RARα gene in AML3 cells by all-*trans* RA might be one of the initial steps restoring the granulocytic differentiation process.

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